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Halobacterial Flagellins Are Sulfated Glycoproteins*

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The cell-surface glycoprotein of *Halobacteria* contains oligosaccharides of the type Glc4→1GlcA4→1GlcA4→1GlcA (where GlcA indicates glucuronic acid) with a sulfate group attached to each of the GlcA residues. We report here that in addition to this cell-surface glycoprotein, the halobacterial flagellar proteins (recently described by Alam, M., and Oesterhelt, D. (1984) *J. Mol. Biol.* 176, 459–475) also contain the same type of sulfated oligosaccharides. These flagellins have the following features. 1) All of the individual flagellar proteins contain identical sulfated saccharide moieties linked to the amido nitrogen of Asn through a Glc residue (the novel type of *N*-glycosidic linkage that has been found in the cell-surface glycoprotein from *Halobacteria* (Wieland, F., Heitzer, R., and Schaefer, W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 5470–5474)). 2) The amino acid sequence of one carbohydrate-binding region is Gln-Ala-Ala-Gly-Ala-Asp-Asn-Jle- Asn -Leu-Thr-Lys. This surrounding sequence is consistent with the general formula Asn-*X*-Thr(Ser), common to all *N*-linked glycopeptides determined so far. 3) Biosynthesis of flagellar glycoconjugates involved sulfated oligosaccharides linked to dolichol monophosphate. 4) The individual glycoproteins making up the flagella are structurally closely related to one another.

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Halobacteria were the first bacteria shown to contain a true glycoprotein (1). Subsequent work revealed that this glycoprotein contains sulfated saccharides in addition to neutral disaccharides (2–4). Biosynthesis of these sulfated saccharides has turned out to exhibit unusual features (5). (i) A dolichol, rather than a bacterial undecaprenol, serves as the lipid anchor to which the oligosaccharides are linked. (ii) Linkage of the oligosaccharides is via a monophosphate bridge rather than through pyrophosphate. (iii) The sulfate residues are present in the oligosaccharides at the lipid-linked level rather than to be introduced at the protein-linked level. (iv) Transient methylation of the 3-position of a peripheral glucose residue is an obligatory step before transfer of the saccharide moiety to the acceptor protein (6).

In addition to the sulfated cell-surface glycoprotein, we have observed a set of heterogenous sulfated proteins, all with molecular masses around 25 kDa (5, 7).

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Recently, Alam and Oesterhelt (8) isolated halobacterial flagella and analyzed their protein composition by SDS[1]-gel electrophoresis. The protein pattern they obtained looked strikingly similar to the above-mentioned pattern of sulfated proteins, and this prompted us to investigate whether these sulfated proteins represent the halobacterial flagellins.

EXPERIMENTAL PROCEDURES[2]

RESULTS

The Polypeptide Portions of Flagella and of Sulfated Proteins Are Identical—When purified flagella from strain M 175 (an overproducer of flagella (8)) were separated by SDS-gel electrophoresis, they yielded a pattern of bands very similar to the set of sulfated proteins with molecular weights around 25,000 that we had obtained earlier after *in vivo* $^{35}SO_4^{2-}$-labeling of strain R_1M_1 (5, 7). These sulfated proteins will henceforth be referred to as components I, II, and III (C I–III). Although the array of the bands was similar in both strains, the entire set of bands derived from M 175 migrated faster than did the sulfated components I–III from R_1M_1 (see Fig. 2, *lanes A* and *B*). Immunological experiments, however, indicated that the polypeptides from the two strains were related to one another. Component I (purified from strain R_1M_1 to homogeneity as described under "Experimental Procedures," see Fig. 3*A*, *lane a*) was solvolyzed with anhydrous HF (4, 15) (see Fig. 3*A*, *lane b*) and used to raise antibodies in a rabbit. Probing component I and SDS-denatured flagella from strain M 175 with this antiserum yielded fusing precipitin bands, indicating immunological identity of their polypeptide chains, as is shown in Fig. 3*B*. Further evidence that component I is a constituent of halobacterial flagella was obtained by immunofluorescence microscopy: glutaraldehyde-fixed *Halobacteria* (prepared as described under "Experimental Procedures") were reacted with the rabbit antibodies against HF-treated component I and, after washing, incubated with fluorescent anti-rabbit-IgG. The result is shown in the fluorescence micrograph in Fig. 4. Distinct fluorescing dots can be observed at the poles of almost every bacterial rod (*short arrows*), and in some cases, fluorescent protrusions can be observed at the poles (*long arrows*). These dots and protrusions most probably represent parts of the flagella, the rest of which had been sheared by the vigorous washing required

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; GlcA, glucuronic acid; OD, optical density; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

[2] Portions of this paper (including "Experimental Procedures" and Fig. 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2130, cite the authors, and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

during preparation of the samples.

Final proof of identity at the polypeptide level of flagellins from strain M 175 and the sulfated components was obtained by peptide mapping: isolated [^{35}S]methionine-labeled components I from strain R_1M_1 as well as isolated ^{35}S-labeled major flagellin from strain M 175 (the component of lowest apparent molecular weight, see Fig. 2, *lanes C* and *D*) were digested with thermolysin and separated by chromatography (first dimension) and high voltage electrophoresis (second dimension). Fig. 5, *A* and *B*, shows a comparison of the resulting [^{35}S]methionine peptide maps: almost identical patterns were obtained. Thus, the immunological result indicating a close relationship of flagellins with the sulfated component I (Fig. 3) was confirmed by protein chemical methods.

When the antiserum directed against deglycosylated component I was probed with isolated components II and III, an unexpected result was obtained: both samples gave precipitin lines fusing with the line caused by component I and fusing with each other (see Fig. 3). This indicated close structural relationship of these proteins, and therefore they were compared by peptide mapping as well. The fingerprints obtained

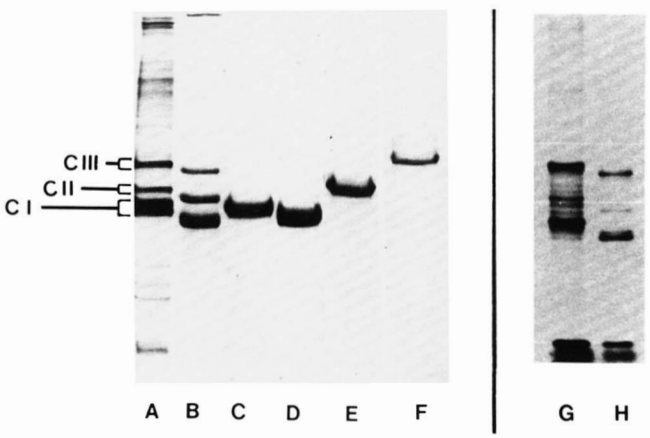

FIG. 2. **Analyses on SDS-polyacrylamide (12% (w/v)) gels of different preparations of sulfated components from strain R_1M_1 as well as flagellins from strain M 175.** *Lane A*, fractions pooled according to Fig. 1C after *in vivo* [^{35}S]methionine labeling of halobacterial strain R_1M_1. *Lane B*, same as in *A*, except that strain M 175 was used. *Lane C*, [^{35}S]methionine-labeled component I (strain R_1M_1) isolated from a preparative SDS-gel for peptide mapping. *Lane D*, same as in *C*, but major flagellin (of lowest apparent molecular weight) from strain M 175. *Lane E*, [^{35}S]methionine-labeled component II. *Lane F*, [^{35}S]methionine-labeled component III. *Lane G*, membrane fraction from strain R_1M_1 after *in vivo* labeling with $^{35}SO_4^{2-}$ according to Ref. 5. *Lane H*, Membrane fraction from strain M 175 after labeling with $^{35}SO_4^{2-}$ according to Ref. 5.

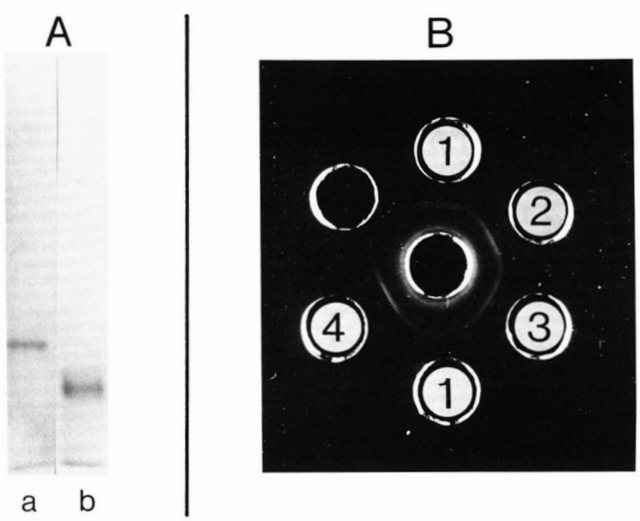

FIG. 3. **Immunodouble-diffusion of different isolated components with an antiserum raised against deglycosylated component I.** *A*, SDS-gel electrophoresis of component I before (*lane a*) and after (*lane B*) deglycosylation with anhydrous HF. The sample shown in *lane b* was used to raise antibodies in a rabbit. *B*, double-diffusion according to Ouchterlony. The central well contained the antiserum against deglycosylated component I. The peripheral wells contained: *1*, component I; *2*, flagellins isolated from strain M 175 according to Ref. 8; *3*, component II; and *4*, component III.

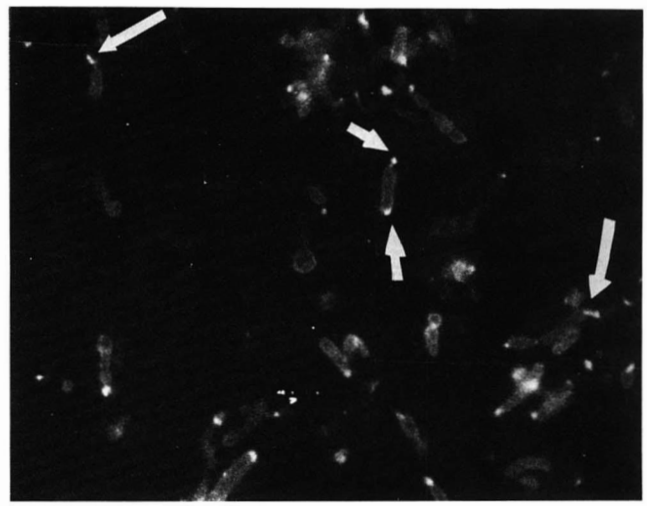

FIG. 4. **Fluorescence micrograph of fixed *Halobacteria* strain R_1M_1 after double-immunostaining with rabbit anti-component I antibodies and 5-isothiocyanatofluorescein-linked anti-rabbit antibodies.** Magnification about 500-fold.

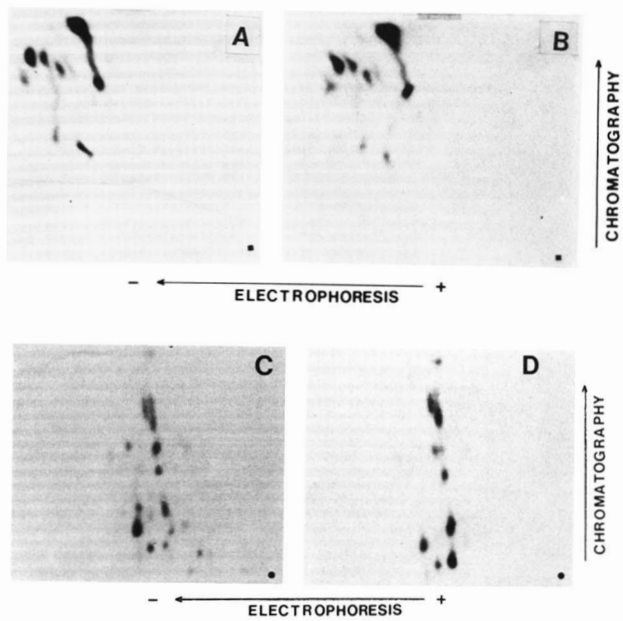

FIG. 5. **Peptide mapping of individual isolated [^{35}S]methionine-labeled proteins.** [^{35}S]Methionine-labeled proteins were isolated from SDS-gels (see Fig. 2, *lanes C–F*) and digested. The resulting peptides were analyzed by chromatography (1st dimension) and high voltage electrophoresis (2nd dimension). The figure shows a fluorograph of the resulting electropherogram. For details see "Experimental Procedures." *A* and *B*, thermolysin-derived peptides from component I from strain R_1M_1 (Fig. 2, *lane C*) and major flagellin from strain M 175 (with lowest apparent molecular weight, Fig. 2, *lane D*) respectively. *C* and *D*, subtilisin-derived glycopeptides from component II (Fig. 2, *lane E*) and component III (Fig. 2, *lane F*), respectively, both from strain R_1M_1.

after digestion with subtilisin of isolated [^{35}S]methionine-labeled components II and III (see Fig. 2, *lanes E* and *F*) are shown in Fig. 5, *C* and *D*. Again similar patterns were obtained.

Taken together, these results show that the set of sulfated components I, II, and III represents the halobacterial flagellins and that the individual flagellins are structurally related to each other.

Individual Flagellar Components Carry Identical Sulfated Glycoconjugates—To investigate the chemical nature of the covalently bound sulfate residues, flagella isolated from strain R_1M_1 after *in vivo* $^{35}SO_4^{2-}$-labeling were digested exhaustively with subtilisin and pronase. After chromatography on Dowex AG 50WX8 H^+ ion exchange resin in water, more than 95% of the original radioactivity was found in the eluate. This material was submitted to methanolysis and analyzed by gas-liquid chromatography-mass spectrometry after perfluoropropionylation. Glc and GlcA were the main components identified. A contaminating amount of Gal occurred, which never exceeded 15% of the Glc content. The sulfated material thus consists of sulfated glycopeptides with a sugar composition very similar (or identical) to that found in the sulfated oligosaccharides derived from the cell-surface glycoprotein from *Halobacteria* (4–6).

For further characterization, subtilisin- and pronase-derived $^{35}SO_4^{2-}$-labeled glycopeptides from individual components I, II, and III were analyzed by high voltage thin layer electrophoresis. In Fig. 6 the corresponding fluorogram is shown: clearly, each of the components gave rise to an identical pattern. Thus, each of the components contains the same types of sulfated glycoconjugates. Moreover, these glycopeptides migrate to positions very similar to those of pronase-derived $^{35}SO_4^{2-}$-labeled glycopeptides from the cell-surface glycoprotein (*cf.* Fig. 6, *A* and *D*).

These data are consistent with the finding that all sulfated proteins in *Halobacteria* share the same pool of sulfated dolichol-linked oligosaccharides as precursors (5, 6).

Characterization of the Carbohydrate-binding Region—The similarity of the sulfated glycopeptides from flagellins to those from the cell-surface glycoprotein suggested that they are also bound to the polypeptide chain via the novel linkage Asn-Glc (4). This could be established in the following experiment. Glycopeptides obtained after digestion of flagellins with subtilisin and pronase as above were dansylated and, after treatment with HF, submitted to reversed phase HPLC. Dansylated material eluting at the position of authentic dansylated Asn-Glc (*arrows* in Fig. 7, *A* and *B*, prepared from the cell-surface glycoprotein) was then submitted to two-dimensional chromatography/thin layer electrophoresis. The resulting fluorescent material co-migrated with authentic dansylated Asn-Glc in both dimensions.

To obtain further insight into the nature of the carbohydrate binding region, a tryptic sulfated glycopeptide from isolated component I was purified using reversed phase HPLC (*cf.* Fig. 8). A sulfated glycopeptide was obtained which had the following composition: 3 Asx, 1 Thr, 1 Glx, 1 Gly, 3 Ala, 1 Ile, 1 Leu, and 1 Lys. The sequence of this sulfated glycopeptide is H_2N-Gln-Ala-Ala-Gly-Ala-Asp-Asn-Ile-*X*-Leu-Thr-Lys-CO_2. The amino acid *X* in position 9 could not be determined, most probably due to the presence of a substituent precluding extraction into the organic phase. From a comparison of the amino acid composition of the sulfated glycopeptide with its sequence, we conclude that amino acid *X* in position 9 is Asn, linked to the sulfated carbohydrate moiety.

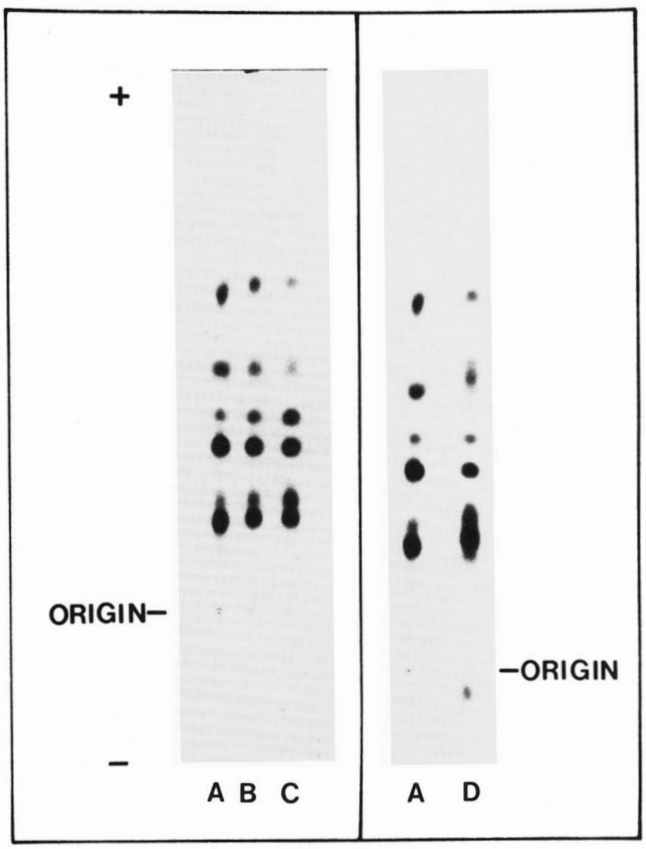

FIG. 6. **Analytical high voltage electrophoresis of $^{35}SO_4^{2-}$-labeled glycoproteins.** $^{35}SO_4^{2-}$-Labeled glycoproteins from halobacterial strain R_1M_1 were digested with subtilisin and then with pronase (for details see "Experimental Procedures"). The resulting glycopeptides were submitted to high voltage thin layer electrophoresis at pH 2. Corresponding fluorograms are shown. The glycopeptides were derived from component I (*lane A*), component II (*lane B*), component III (*lane C*), and the cell surface glycoprotein (fraction of low molecular weight-sulfated glycopeptides according to Ref. 4). (*lane D*).

DISCUSSION

The above data demonstrate that flagella from the halobacterial strain R_1M_1 consist of a set of glycoproteins with sulfated oligosaccharides. This finding corrects a previous assumption that these three sulfated components are structurally related to bacteriopsin (7). $^{35}SO_4^{2-}$-Labeled flagella from strain R_1M_1 on SDS gels co-migrate with $^{35}SO_4^{2-}$-labeled components from halobacterial wild type (not shown here).

The sulfated saccharides are linked via glucose to asparagine residues in the individual flagellins. The amino acid sequence around the *N*-glycosidic linkage of a sulfated glycopeptide is Asn-Leu-Thr, which is consistent with the formula

Asn(CHO)-*X*-Thr(Ser)

common to all sequences around *N*-glycosidic linkages determined so far. Similar, but not identical sequences have been found around the linkage Asn of sulfated glycopeptides from the cell-surface glycoprotein of *Halobacteria*: - Asn(CHO) -Ser-Ser- and - Asn(CHO) -Ala-Ser (data not shown).

The gycoconjugates are of the type Asn-Glc1→4GlcA1→4GlcA1→4Glc and Asn-Glc1→4GlcA1→4GlcA1→GlcA, as deduced by comparison with sulfated glycoconjugates of known structure from the cell-surface glycoprotein (4, 5).

Additional evidence that this type of carbohydrate is com-

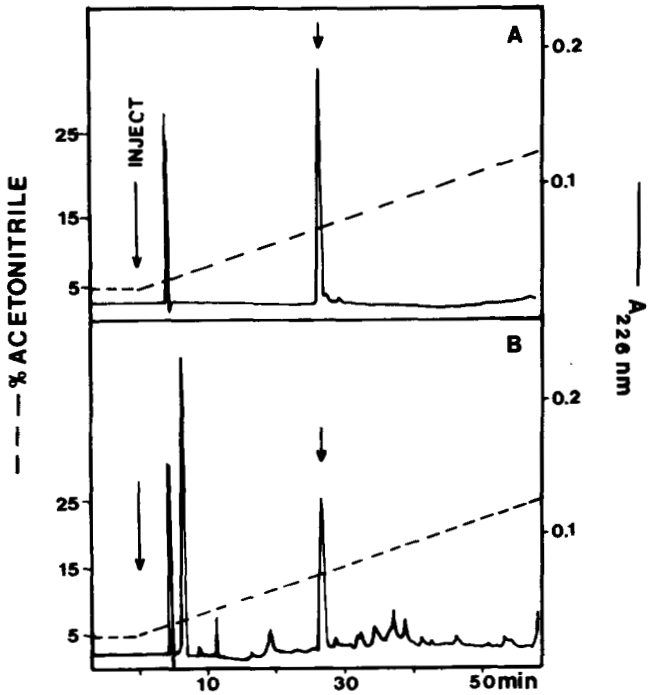

FIG. 7. **Identification of Asn-Glc in halobacterial flagella.** *A*, isolation of authentical dansylated Asn-Glc by HPLC on a reversed phase RP 18 column (for details see "Experimental Procedures"). About 10 μg of the compound Asn-Glc (isolated from the halobacterial cell-surface glycoprotein according to Ref. 4) was dansylated and submitted to HPLC. The flow rate was 1 ml/min, and a fraction was taken every minute. The fraction indicated with an *arrow* was dried and used for two-dimensional separation. *B*, subtilisin- and pronase-derived glycopeptides from strain M 175 flagellins after dansylation were submitted to HPLC. Conditions were those under *A*. The fraction indicated with an *arrow* was further analyzed.

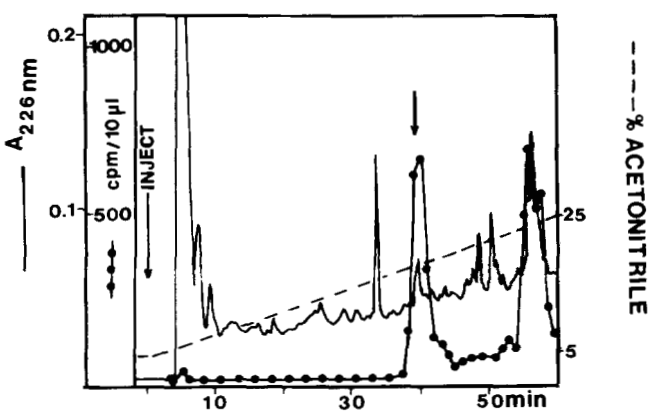

FIG. 8. **Isolation of a $^{35}SO_4^{2-}$-labeled tryptic glycopeptide from component I (from strain R_1M_1).** A tryptic digest of $^{35}SO_4^{2-}$-labeled component I was submitted to reversed phase HPLC as described under "Experimental Procedures." The flow rate was 1 ml/min, and fractions containing 1 ml were collected. The radioactivity was determined in 10 μl of each fraction. The two fractions indicated with an *arrow* were combined and used for amino acid analysis and sequence determination. Details are given under "Experimental Procedures."

mon in *Halobacteria* comes from the fact that all sulfated proteins in this organism share a common pool of sulfated dolichol-oligosaccharide precursors which were all characterized in detail previously (5, 6). Preliminary results indicate that each of the GlcA residues in the carbohydrates is esterified with sulfuric acid at its 2-position.

On SDS gels the flagella display a ladder-like pattern of different bands with three centers of intensity (see Fig. 2, *lanes G* and *H*). Since $^{35}SO_4^{2-}$-labeled glycopeptides from components I–III all yielded identical patterns on high voltage electrophoresis (*cf.* Fig. 6, *lanes A–C*), this heterogeneity of the flagellins most probably is due to different degrees of glycosylation. Accordingly, each of the individual rungs of the ladder therefore is likely to represent one of three related proteins containing a different number of sulfated glycoconjugates.

The proteins from flagella of the overproducing mutant strain M 175 showed a pattern on SDS gels with maxima of intensity shifted towards lower apparent molecular weights (*cf.* Fig. 2, *lanes B* and *H*). We suppose that the mutant polypeptides have lost one or more sites of glycosylation. If correct glycosylation of the flagellins is necessary for proper incorporation of the flagella into the halobacterial cell envelope, the phenomenon of overproduction in this mutant could easily be explained: imperfectly integrated flagella dissociate into the medium, thus causing the need for continuous biosynthesis of new flagellins.

In general, bacterial flagellar filaments are aggregates of only one type of flagellin. As an exception, the filament of flagella from *Caulobacter* is made up from two flagellins which differ slightly in molecular weight (18). Eucaryotic flagella, on the other hand, are composed of a complex variety of different proteins.

Halobacteria, which belong to the kingdom of *Archaebacteria*, possess flagella with filaments made up of three different flagellins and thus exhibit a degree of complexity in between *Eubacteria* and eucaryotes. These flagellins are sulfated glycoproteins. To our knowledge, so far, no other glycosylated flagellins have been described.

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Supplementary Material to "Halobacterial Flagellins are Sulfated Glycoproteins" by Felix Wieland, Gerhard Paul and Manfred Sumper.

EXPERIMENTAL PROCEDURES

General Methods

Quantitative as well as qualitative sugar analyses, either by colorimetric assay or by gas liquid chromatography (GLC), were performed as described (5). Uronic acids were analyzed by GLC and GLC-mass spectroscopy (GLC-MS) as outlined in (5). Reduction of uronic acids with sodium [^{2}H]borohydride according to (9) is also described in (5). Sulfate was determined after hydrolysis of samples at 105°C for 4 h in 6N HCl according to (10). Amino acid analyses: amino acids were determined with an automated amino acid analyzer (LC 5000, Biotronic, FRG) after hydrolysis of samples in constant boiling HCl at 105°C for both 12 and 24 h.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS gel electrophoresis) in gels containing 6 or 12 % (w/v) acrylamide and 4 % (w/v) N,N'-methylene bisacrylamide was performed according to (11).

Fluorography of the gels was performed according to (12) and autoradiography was performed without pretreatment of the gels.

Isolation of radioactively labeled proteins: Regions of interest were cut out of the dried gel using the autoradiogram as a guide. The pieces were soaked in H_2O, pressed through a steel net (about 200 mesh), and the resulting gel slurry was then shaken for 60 min at 4°C in water, and centrifuged. The supernatants (typically containing between 50 and 70% of the radioactivity) were lyophilized, resuspended in water, and separated from gel buffer constituents by gel filtration on Sephadex G 10 (Pharmacia).

Special Procedures

$^{35}SO_4^{2-}$ and [^{35}S]methionine labeling: in vivo labeling of a wild type halobacterial strain, as well as of the mutants R_1M_1 (gas vacuole$^-$ and bacterioruberin$^-$)and M 175 (an overproducer of flagella, generously supplied by Dr. Oesterhelt, München) with $^{35}SO_4^{2-}$ was essentially as described (5). For in vivo [^{35}S]methionine labeling of flagella from strain M 175, bacteria were grown for 3 to 5 days in complex medium (7) in the presence of 4 µCi/ml of [^{35}S]methionine (800 Ci/mMol, Amersham). Flagella were then isolated from the culture supernatant according to (8). For in vivo labeling of R_1M_1 with [^{35}S]methionine, cells were grown to give a final OD at 578 nm of 0.8 to 1 (an OD of 1 at 578 nm corresponding to a cellular protein concentration of 0.5 mg/ml), washed with complete basal salts (3) and shaken for 4 h at 39°C at the original cell concentration in basal salts containing 0.5% (w/v) alanine. The cells were then centrifuged and resuspended in basal salts/0.5% (w/v) alanine to give a final OD at 578 nm of 15. 100 µCi [^{35}S]methionine (800 Ci/mMol, Amersham) were added per 1 ml, and the suspension incubated for 30 min as described in (7), with gentle stirring at 39°C under illumination.

Proteolytic digestions: exhaustive digestion of radiolabeled proteins isolated from gels as described above, was performed on glass fiber filters (Whatman, GFC) in a volume of 40 µl according to (7). After the addition of 50 µg unlabeled bacterioopsin as a carrier, 5 µg subtilisin (Sigma) was added and the sample incubated at 39°C for 12 h. Digestion was stopped by heating to 96°C for 3 min, and subsequently the sample was further digested at 56°C for 4 h by the addition of 5 µg Pronase E (Serva).

Digests were analyzed by high voltage electrophoresis on thin layer sheets (20 x 40 cm Macherey & Nagel, Düren, FRG) in formic acid/acetic acid/water (2:8:90, by volume, pH = 2.0).

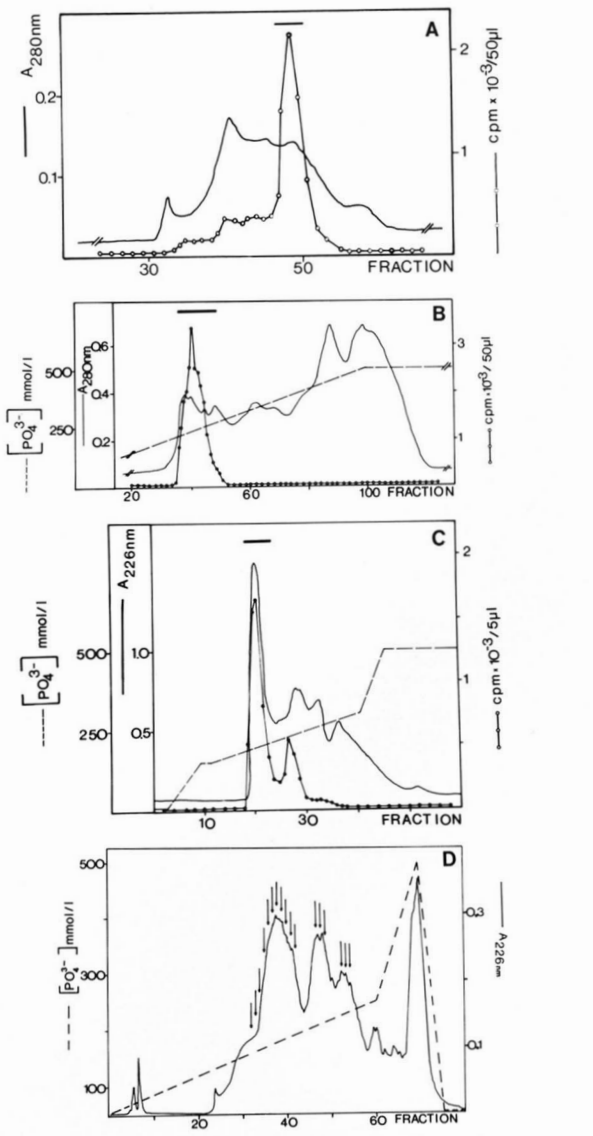
Fingerprint analyses: [^{35}S]methionine labeled samples extracted from gels were precipitated with trichloroacetic acid, and after addition of 50 µg of bacterioopsin as a carrier, applied to glass filters, and washed as described in (7). After incubation with 4 µg thermolysin (Sigma) in 40 µl of 0.1 N N-methylmorpholinoacetate buffer pH 7.5 at 39°C for 12 h, the resulting [^{35}S]methionine-labeled peptides were analyzed by two dimensional thin layer chromatography-high voltage electrophoresis (first dimension: chromatography in pyridine/butanol/H_2O (35:35:30), second dimension: electrophoresis for 50 min at 50 V/cm in acetic acid/formic acid/H_2O (8:2:90 by volume, pH 2.0). In this system all of the [^{35}S]methionine-labeled peptides migrated towards the anode. Fluorography of the electropherograms was performed according to (13).

Demonstration of Asn-Glc in a pronase digest of flagellar proteins: after purification on a HPHT-column, cell surface glycoprotein-free flagella proteins (isolated as described in this section, purity being assessed by SDS gel electrophoresis) were digested with substilisin followed by pronase as described above, and passed through a small column of Dowex 50 WH$^+$ (200-400 mesh) ion exchange resin (Bio Rad) to separate the sulfated glycopeptides from residual peptide material. The eluate (containing about 90% of the sulfated glycopeptides) was dansylated according to (14).

The mixture of dansylated sulfated glycopeptides was treated with anhydrous HF according to (4,15) and submitted to HPLC on a reversed phase column (RP 18, 10 µm, Lichrosorb, Merck). A gradient of acetonitrile containing 0.1 % trifluoroacetic acid was applied from 5% acetonitrile to 25 % in 60 min. The dansylated material eluting with the retention time determined for authentic dans-Asn-Glc was collected, lyophilized and submitted to two dimensional chromatography/high voltage electrophoresis (first dimension: chromatography as above second dimension: high voltage thin layer electrophoresis in pyridine/acetic acid/water (2:1:197 by volume; pH 5.5) at 50 V/cm for 40 min.

Isolation of CI, II and III from strain R_1M_1: *Halobacterium halobium* strain R_1M_1 was grown in complex medium in a 50 l fermentor (Braun, Melsungen) at 39°C with aeration (25 l air/min) and stirring (300 rpm). After 5 days, an OD of 1.2 to 1.8 at 578 nm was reached,and the cells harvested using a milk homogenizer centrifuge (Westfalia, Dortmund). For lysis, cells (120-160 g wet weight) were stirred with a Sorvall Omni-Mixer (LKB, Bromma, Sweden) with 2 liters of ice cold 50 mM Tris/HCl buffer, pH 6.8, for 2 h at 4°C. During this treatment, nucleic acids were sheared mechanically. The resulting suspension of broken cells was centrifuged in a Kontron TFA 20 Rotor at 19000 rpm (about 50 000 x g) for 12 h at 4°C. The resulting pellet (crude membranes) was resuspended in 200 ml of the above buffer and centrifuged through a cushion of 20 % (w/v) sucrose in the same buffer (100 000 x g, 4 h). Lipids were extracted from the resulting pellet as described in (5), with an additional wash with 100 ml of ethanol. The precipitate was dissolved in 20 ml 0,1 M Tris/HCl buffer pH 6.8, containing 50 mM NaCl and 0,1 % SDS, with the further addition of 10 ml of a 10% (w/v) solution of SDS. After addition of $^{35}SO_4^{2-}$-labeled membranes obtained by in vivo $^{35}SO_4^{2-}$-labeling and purification as described above, the sample was submitted to gel permeation chromatography on Sephacryl S 300 (5 x 80 cm, Pharmacia) in 0.1 M Tris/HCl buffer, pH 6.8, containing 50 mM NaCl, 10 mM mercaptoethanol and 0.1% SDS. The eluate was monitored for protein at 278 nm and the radioactivity in the fractions (15 ml) was determined. The elution profiles obtained are shown in figure 1A. Fractions indicated with a bar contained CI-III as assessed by SDS-Gel electrophoresis and subsequent fluorography. These were pooled, and concentrated in an ultrafiltration cell (filter PM 10, Amicon). The concentrated material was applied to a Bio Gel HT-column (2,5 x 25 cm, Bio Rad), equilibrated with 50 mM sodium phosphate buffer pH 6.5, containing 0.1% SDS. A linear gradient of sodium phosphate from 50 mM to 500 mM was applied in an elution volume of 1.8 liters.This step is essential to separate component I from bacterioopsin, which on SDS-gels shows an apparent molecular weight very similar to component I. On the Bio Gel HT column described here, however, bacterioopsin elutes at a much higher phosphate concentration than do the sulfated components. The eluate was monitored at 278 nm and the radioactivity in each of the 18 ml-fractions determined. The resulting elution profile is shown in fig. 1B. Fractions containing CI-III, indicated with a bar, were again pooled. After concentration by ultrafiltration, desalting by resuspension in water and reconcentration on the ultrafilter, the material was rechromatographed on a HPLC hydroxylapatite column (HPHT, BioRad)under the conditions described in the legend to fig. 1. The resulting elution profile is shown in fig. 1C. Fractions of interest (indicated with bars) were pooled, and after desalting and concentration they were submitted to preparative SDS-gel electrophoresis and isolated from the gels as described above. Component I was then deglycosylated with anhydros HF (90 min/0°C) (4,15) and used to raise antibodies in a rabbit.

Flagella from mutant strain M 175 were isolated according to (8). Partial separation of the components I, II, and III from strain R_1M_1 as well as individual flagellins from flagella isolated from strain M 175 according to (8) could be achieved by chromatography on the HPHT column under the conditions indicated in the legend to fig. 1D, which shows the elution profile so obtained. Fig. 1E shows the protein pattern obtained after SDS gel electrophoresis of individual fractions (indicated with arrows in fig. 1D).



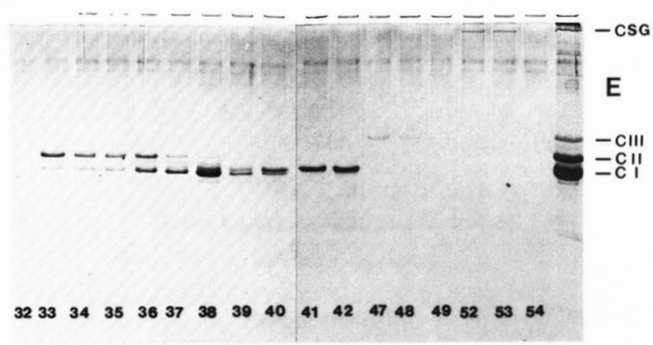


Figure 1
Individual steps during isolation of component I and individual flagellins. For details see "Experimental Procedures".
A: Elution profile obtained after gel filtration of one third of the delipidated material yielded from a 50 l fermentation. Fractions were pooled as indicated with a bar. B: Elution profile after chromatography of the pool in fig. 1A on Bio Gel HT (BioRad). Fractions were pooled as indicated with a bar and desalted as described under "Experimental Procedures". C: Elution profile obtained after HPLC of the pool in fig. 1B on a HPHT-column (BioRad). Gradient: 50-300 mM sodium phosphate pH 6.5, 0.1 % SDS, 60 min, with a flow of 0.5 ml/min. 2 min-fractions (1 ml) were collected and pooled as indicated with a bar. D: Elution profile obtained after HPLC on a HPHT column of a crude flagella fraction isolated from strain M 175 according to (8). Gradient: 25-225 mM sodium phosphate pH 6.5, 0.1 % SDS, 120 min, with a flow of 0.5 ml/min. 2 min-fractions(1 ml) were collected. E: Analytical SDS-gel electrophoresis on a 12 % (w/v) polyacrylamide gel of the fractions indicated with arrows in fig. 1D (Coomassie staining). Numbers refer to the fractions investigated. The unnumbered lane (right) shows the flagella-preparation before chromatography. CSG: cell surface glycoprotein.

Isolation of a tryptic glycopeptide: 500 µg of isolated $^{35}SO_4^{2-}$-labeled component I was incubated with 10 µg of TPCK-treated Trypsin (Sigma) in 0.1 M N-methylmorpholine acetate buffer pH 7.5, containing 1 mM $CaCl_2$, at 37°C for 3 h. After addition to a further 10 µg of protease, the sample was incubated for 12 h at 37°C. The digest was then passed through a small column of AG 50 W x 8 H^+ ion exchange resin with water as eluant. More than 90 % of the radioactivity was found in the aqueous eluate, which then was submitted to reversed phase HPLC (Lichrosorb, RP 18, 10 µm, 0.4 x 25 cm, Merck) using a gradient of acetonitrile in 0.1 % trifluoroacetic acid. Fractions containing radioactivity were rechromatographed. Sequence determinations were performed by Dr. Lottspeich, Martinsried, on an Edman spinning cup automated sequence analyzer. PTH-amino acids were analyzed by HPLC according to (16,17).

Preparation of "fixed" Halobacteria: Cells grown in complete medium were harvested by centrifugation, and resuspended in basal salts to give a final OD of 3,0 at 578 nm. Glutaraldehyde was added to give a final concentration of 2 %. After incubation at 37°C for 60 min cells were sedimented by centrifugation and washed twice in basal salts containing 10% (w/v) glycine. The cells were then washed twice in 0.2 M NaCl, 0.2 M KCl, 0.1 M Tris/HCl, pH 8.0 (Buffer A), containing 10% (v/v) of goat serum. The fixed bacteria were resuspended in saline (10 mM Tris/HCl pH 7.5, 0,9% NaCl, 0.1% NaN_3) to give a final OD of 3.0 at 578 nm.

Preparation of fluorescently-labeled samples: 250 µl of a suspension of fixed Halobacteria in saline were shaken at 20°C with 20 µl of the rabbit anti-component I-serum for 30 min. The sample was then washed 3 times with Buffer A-containing goat serum (10% (v/v)) and resuspended in 250 µl saline. To this solution 3 µl of FITC-linked anti-rabbit-antibody (whole molecule, Sigma) was added. After incubation for 30 min at 20°C with shaking, the sample was washed as follows: twice with 500 µl Buffer A/goat serum (10% (v/v)), three times with 1 ml of Buffer A, and twice with saline. After resuspension in 250 µl saline, the sample was observed in a fluorescence - microscope (Leitz, magnification about 500-fold).